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Indirect Immunofluorescence Assay for Detection of *Helicobacter pylori* in Human Gastric Mucosal Biopsies

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To determine sensitivity and specificity of immunofluorescence assay (IFA) for detection of *Helicobacter pylori*, we studied 151 patients. Biopsies of gastric mucosae were obtained for culture, histological testing, and IFA. *H. pylori* serum antibodies were tested by enzyme-linked immunosorbent assay. IFA was done on Formalin-preserved, paraffin-embedded biopsies by using rabbit anti-*H. pylori* and goat anti-rabbit gamma globulin-fluorescein isothiocyanate conjugate. The sensitivity and specificity of IFA compared with culture and Warthin-Starry stain were 93 and 95%, respectively. IFA is an accurate method for the diagnosis of *H. pylori* infection.

Helicobacter pylori (formerly *Campylobacter pylori*) (2) causes 97% of gastritis cases (5) and is associated with human duodenal ulcers 100% of the time (3). Specific diagnosis of *H. pylori* infection requires culture of the gastric mucosal biopsy in selective media. Indirect immunofluorescence assay (IFA) has been used successfully in the diagnosis of infections caused by fastidious organisms and has proved useful to identify *H. pylori* in pure cultures (1) and in gastric mucosal smears (7), but its application for direct diagnosis in routine Formalin-preserved, paraffin-embedded biopsies has not been described.

To evaluate IFA as a diagnostic method and to determine its sensitivity and specificity in the detection of *H. pylori* infection, we studied patients aged 16 to 75 years who underwent endoscopy for the investigation of upper gastrointestinal symptoms from February to August 1988 at the National Institute of Nutrition in Mexico City, Mexico. Patients were excluded if they had received antimicrobial agents or bismuth salts the previous week, if they had any conditions which may hinder the isolation of *H. pylori*, such as recent gastric bleeding, or if they were immunosuppressed.

Five forceps biopsies were obtained from each of the 151 consenting patients who were eligible. The first two specimens were placed in 1 ml of 0.9% sterile saline solution at 4°C and were transported to the laboratory within 2 h. The tissue was processed through a glass tissue grinder and plated onto brain heart infusion agar supplemented with 1% IsoVitaleX (Bioxon Laboratories, Mexico City, Mexico), 7% defibrinated horse blood, and selective antibiotics (vancomycin [6 mg/liter], trimethoprim [20 mg/liter], and polymyxin B [5 mg/liter]; Sigma Chemical Co., St. Louis, Mo.). Plates were incubated for at least 7 days in anaerobic jars in a microaerophilic environment (10% CO_2, 85% N_2, and 5% O_2) at 37°C. Macroscopically, *H. pylori*-like colonies were further identified by Gram stain and urease and oxidase tests. The urease test was performed in the third specimen as previously described (8) by incubating it in 0.5 ml of Christensen urea broth. The last two specimens were preserved in 10% Formalin and were processed and stained with hematoxylin and eosin and Warthin-Starry (W-S) stains. Slides were observed by a pathologist who ignored the clinical, microbiological, and serological results.

In addition to the biopsies, 10 ml of blood was obtained from each patient for detection of specific serum *H. pylori* antibodies by enzyme-linked immunosorbent assay (ELISA) by using the glycine acid extract proteins as antigen as previously described (3). In our experience, this test is 91% sensitive and 95% specific compared with culture and W-S stain.

For the IFA, *H. pylori* antiserum was produced in New Zealand rabbits by subcutaneously injecting 10^{10} CFU of formalinized bacteria (*C. pylori* CCUG 15818, kindly provided by E. Falsen from University of Göteborg, Göteborg, Sweden) plus Freund's complete adjuvant (equal volume), followed by weekly subcutaneous injections of 10^{10} CFU of formalinized bacteria for 3 consecutive weeks. The animals were bled by heart puncture 1 to 2 weeks later. Serum titers were determined by ELISA by using the glycine acid extract surface proteins as antigen; the final titer was 1:3,250. As determined by checkerboard titration, the optimal dilution of rabbit *H. pylori* antiserum was 1:100, and that of fluorescein isothiocyanate–anti-rabbit gamma globulin conjugate (Cappel Laboratories, Cochranville, Pa.) was 1:400; both were diluted in bovine serum albumin–phosphate-buffered saline (BSA-PBS).

The specificity of *H. pylori* antiserum was determined by IFA and ELISA. The serum was absorbed with *Campylobacter jejuni*, *Campylobacter fetus*, *Escherichia coli*, *Pseudomonas* spp., and *H. pylori* by incubating with a suspension of 10^9 CFU for 18 h at 4°C. Slides of a culture-positive biopsy were then processed by IFA. Competitive inhibition ELISA was done by adding increasing concentrations from 0.04 to 30 μg of homologous and heterologous antigens per ml until the homologous inhibition was >50%.

IFA was done on two slides of Formalin-preserved, paraffin-embedded tissue from each patient. Slides were washed with xylol-alcohol. To avoid nonspecific adsorption, slides were incubated with 1% BSA-PBS and washed three times

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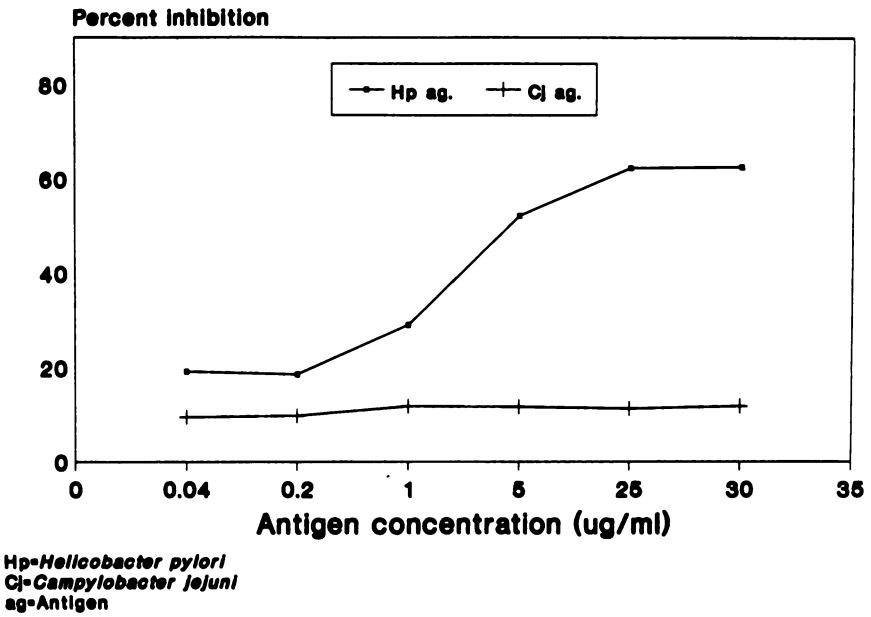

FIG. 1. Inhibition of binding of anti-*H. pylori* antibody to solid-phase-bound *H. pylori* antigen in ELISA by different concentrations of glycine acid extract proteins of *H. pylori* (■) or *C. jejuni* (□). The initial concentration of inhibitors was 35 μg of glycine acid extract proteins of *H. pylori* and *C. jejuni* per ml.

with PBS. Slides were incubated for 60 min with the specific anti-*H. pylori* rabbit serum diluted 1:100 in 0.1% BSA-PBS and washed with PBS. Goat serum anti-rabbit gamma globulin–fluorescein isothiocyanate conjugate diluted 1:400 in 0.1% BSA-PBS was added and incubated for 90 min. The slides were sealed and blindly observed with an epifluorescence microscope (Axioskop no. 451485; Karl Zeiss) by two independent observers. IFA was considered positive when at least three bacteria fluoresced with the same intensity as the controls in at least two biopsies.

Reproducibility of IFA was evaluated by concordance between observers of the 151 slides and by intraobserver agreement for at least 20 slides. Sensitivity and specificity of IFA were calculated by comparing the results with culture or W-S stain as a reference standard.

A total of 151 patients were studied, 79 women and 72 men with mean ages of 38.9 and 46.6 years, respectively. There were 87 (57.24%) *H. pylori*-positive cultures. Ninety-six (63.5%) urease tests were positive; 53 (35%) were positive in less than 4 h, 27 (18%) were positive in the following 4 h, and the rest were positive in less than 24 h. *Campylobacter*-like structures were seen by W-S stain in 125 (82.7%) biopsies, and ELISA for *H. pylori* was positive for 120 (79.4%) patients.

IFA specificity assays showed no cross-reactivity; when the hyperimmune serum was absorbed with *C. jejuni*, *C. fetus*, *E. coli*, or *Pseudomonas* spp., the intensity of fluorescence remained stable compared with that of the nonabsorbed serum. In contrast, when serum was absorbed with the *H. pylori* whole-bacterium suspension, the fluorescence disappeared.

In the competitive inhibition ELISA, no significant inhibition of *H. pylori* hyperimmune serum was observed when *C. jejuni* (Fig. 1), *C. fetus*, *E. coli*, or *Pseudomonas* antigens were added, even when concentrations of up to 30 μg/ml were used. In contrast, when *H. pylori* antigen was added at a concentration of 5 μg/ml, there was a 52% inhibition, which increased to 62% when the concentration was increased to 30 μg/ml.

H. pylori IFA was positive in 122 of 151 (81%) samples. Of these, 86 (70.4%) had positive cultures, 95 (77.8%) had positive urease tests, 116 (95%) had positive W-S stains, 105 (86%) had histological evidence of gastritis, and 109 (89%) had *H. pylori*-specific antibodies. Of the biopsies negative by IFA, only one was positive by culture; *H. pylori* was frequently observed in the mucus over the gastric epithelial cells and often inside the crypts by IFA and W-S stain.

The sensitivity of IFA was 93.1% compared with culture or W-S stain. The specificity was 95% since only one biopsy was positive for IFA of the 21 biopsies which were negative by culture, W-S stain, and urease test (Table 1). The reproducibility of IFA showed a kappa value for concordance between observers of 0.79, and the kappa value for intraobserver agreement was 0.88. Figure 2 shows a touch-imprint biopsy positive for IFA and fluorescent bacteria near the gastric mucosa.

We found a prevalence of *H. pylori* infection of 86% among our patients, although histological changes of gastritis

TABLE 1. Parameters of IFA for the diagnosis of *H. pylori* infection[a]

Parameter	% (no./total)
Sensitivity	93 (121/130)
Specificity	95 (20/21)
Positive predictive value	99.1 (121/122)
Negative predictive value	68.9[b] (20/29)
Accuracy	94.0 (142/151)

[a] Compared with culture or W-S stain. The 95% confidence intervals for sensitivity and specificity were 92 to 96 and 93 to 97, respectively.
[b] Prevalence = 86%.

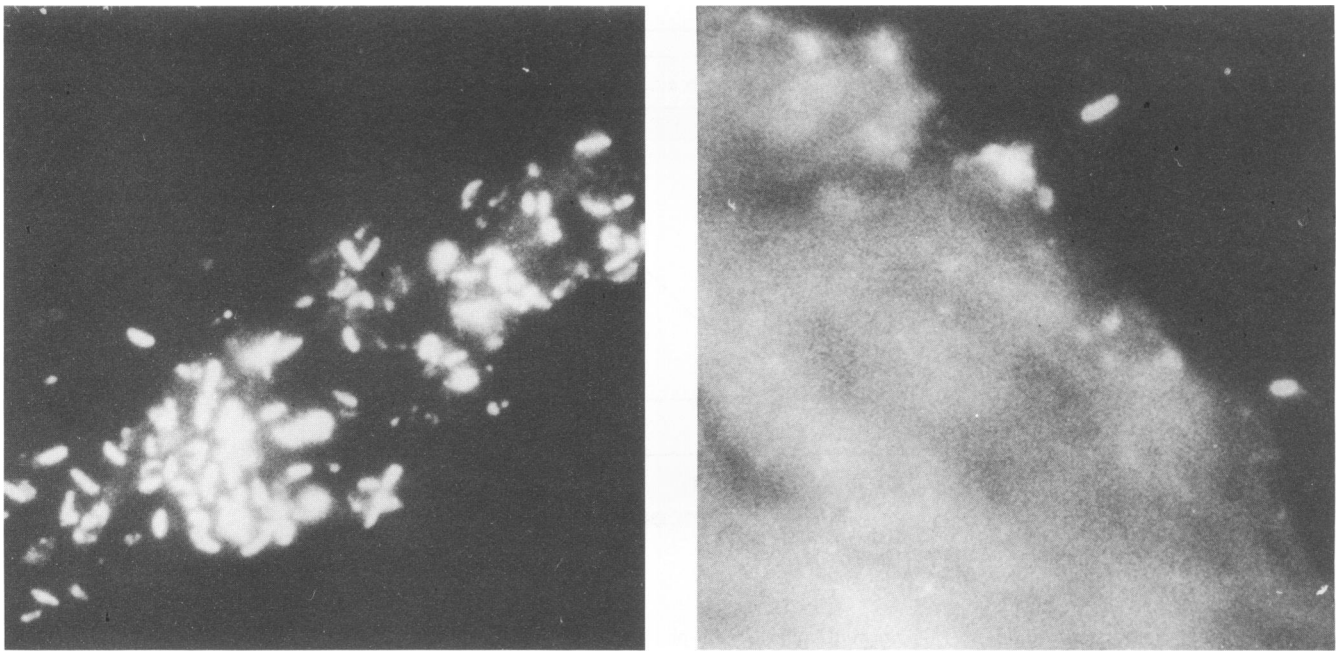

FIG. 2. (A) Touch-imprint biopsy positive for *H. pylori* by IFA. (B) Immunofluorescence assay of a biopsy positive for *H. pylori*. Fluorescent bacteria are seen near the gastric mucosa (epifluorescence magnification is ×1,000).

were present in 96% of infected patients, as others have reported (6). In previous studies comparing results of several stains such as W-S, hematoxylin-eosin, Gram, and Giemsa on sections of the same tissue specimen (4), it was concluded that *H. pylori* cells were consistently easier to identify with W-S stain. Although these methods were more sensitive than culture, the specificity was around 78% (4). Unfortunately, the primary isolation of *H. pylori* from biopsies of gastric mucosae is a difficult procedure that takes 3 to 7 days and is frequently unsuccessful because of the fastidious growth of *H. pylori*. Of 125 samples positive by W-S stain, 82 (65%) had positive cultures; these data are comparable with those of Schaber et al., who reported 55% positive cultures. The need for a final diagnosis requires the detection of *H. pylori*. The IFA used in this study provided a fast microscopical method with specific immunological reaction, with a lower rate of false-positive results than the W-S stain. IFA has a higher sensitivity and is faster than culture (6 h). Another advantage is that the antiserum used in this IFA did not cross-react with closely related bacteria, such as *C. jejuni* and *C. fetus*, or nonrelated bacteria. The highest cross-reactivity by inhibition ELISA was 8.3%, as has been reported by Van Bohemen et al. (9) and Goodwin et al. (3), and no cross-reactivity was seen by IFA. Thus, false-positive results are highly unlikely. The *H. pylori* antisera reacted similarly with several *H. pylori* isolates. The low rate of false-negative results by IFA, 6.9%, can be decreased by additional use of the urease test.

Performing the IFA on Formalin-preserved, paraffin-embedded gastric mucosal biopsies allows the evaluation of *H. pylori* infections in collection biopsies with a high degree of sensitivity and specificity. Indirect immunofluorescence in fresh smears of gastric mucosae has showed a higher sensitivity (96%), but its application in collection samples is not possible (7).

The sensitivity, specificity, and reproducibility of our IFA showed that it could be useful as an alternative diagnostic test for *H. pylori*-associated gastritis.

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